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TITLE: ANIMAL MODEL

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ANIMAL MODEL

RELATED APPLICATIONS

- 5 This application is a divisional of application Serial No. 09/587,945, filed June 6, 2000, which is a continuation-in-part of application Serial No. 09/085,380, filed May 26, 1998, and claims priority to U.S. provisional application number 60/190,692, filed March 20, 2000, and Swedish application number 9701963-2, filed May 26, 1997. The disclosures of the prior applications are considered part of (and are incorporated by
10 reference in) the disclosure of this application.

TECHNICAL FIELD

- 15 The invention relates to transgenic non-human mammalian animals being capable of expressing the human FKHL14/FOXC2 gene in its adipose tissue. The invention also relates to methods for identifying compounds useful for the treatment of medical conditions related to obesity or diabetes, said compounds being capable of stimulating expression of the human FKHL14/FOXC2 gene, or being capable of stimulating the
20 biological activity of a polypeptide encoded by the human FKHL14/FOXC2 gene. The invention further relates to methods for identifying compounds useful for the treatment of medical conditions related to malnutrition, said compounds being capable of decreasing expression of the human FKHL14/FOXC2 gene, or being capable of decreasing the biological activity of a polypeptide encoded by the human FKHL14/FOXC2 gene.

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BACKGROUND ART

More than half of the men and women in the United States, 30 years of age and older, are now considered overweight, and nearly one-quarter are clinically obese (Wickelgren, 1998). This high prevalence has led to increases in the medical conditions that often accompany obesity, especially non-insulin dependent diabetes mellitus (NIDDM), hypertension, cardiovascular disorders, and certain cancers. Perhaps most importantly, obesity confers a significant increased rate of mortality when compared with that of individuals of normal body weight. Obesity results from a chronic imbalance between energy intake (feeding) and energy expenditure. Energy expenditure has several major components including basal metabolism, physical activity, and adaptive (nonshivering) thermogenesis. This latter process refers to energy that is dissipated in response to changing environmental conditions, most notably exposure to cold or excessive caloric intake (so-called diet-induced thermogenesis). To better understand the mechanisms that lead to obesity and to develop strategies in certain patient populations to control obesity, we need to develop a better underlying knowledge of the molecular events that regulate the differentiation of preadipocytes and stem cells to adipocytes, the major component of adipose tissue.

Role of adipose tissue

The reason for existence of the adipocyte is to store energy for use during periods of caloric insufficiency. Postprandially, dietary fat is absorbed via the intestine and secreted into the circulation as large triglyceride (TG) rich particles called chylomicrons (chylo). Lipoprotein lipase (LPL), although produced by adipocytes, is localized to the endothelial cell surface where it hydrolyses TG resulting in the release of free fatty acids (FFA). Much of these are taken up by the adipose tissue either passive or active via FFA transporters. The FFAs are then activated to an acyl CoA form and re-esterfied by an enzymatic cascade to form storage TG. At the same time, glucose, which also increases in the circulation postprandially, is taken up into adipose tissue via specific plasma membrane glucose transporters. These two substrates (glucose and FFA) are the building blocks for formation of storage TG. On

the other hand, during fasting, FFAs are released from the adipose tissue TG pool through the action of hormone sensitive lipase (HSL; Fig. 1). Clearly, efficient functioning of adipose tissue is dependent on the coordinated control of each of these processes and the proteins involved.

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In recent years, a growing body of evidence has demonstrated a dual role for adipocytes, also being a source of numerous hormones that regulate both the adipocyte itself and many other systems within the body. Adipocytes produce leptin as a function of adipose energy stores. Leptin acts through receptors in the hypothalamus to regulate appetite, activity of brown adipose tissue (BAT), insulin secretion via sympathetic nervous system output, and important neuroendocrine adaptive responses to fasting and control of reproduction. The gene encoding leptin was identified by positional cloning (Zhang *et al.*, 1994) and is the mutation leading to the profound obese phenotype of the ob/ob mouse, characterized by severe obesity, NIDDM, diminished fertility and hypothermia. The db-gene codes for a hypothalamic receptor for leptin (Chua *et al.*, 1996) and the db/db mutant mice show a similar phenotype with ob/ob mice, but here the defect lies in the block of leptin receptor downstream signaling. After leptin administration, it was possible to correct the defect only in the ob/ob, but not db/db mice as predicted by Coleman's parabiosis experiments (Coleman, 1973).

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Another adipocyte product, the cytokine tumor necrosis factor α (TNF α), has profound effects on adipocyte differentiation, and energy metabolism, and can even induce adipocyte dedifferentiation and apoptosis. Furthermore, TNF α has more systemic implications as it has been shown to play a role in the genesis of insulin resistance associated with obesity (Hotamisligil *et al.*, 1993). In obese humans and numerous rodent models of obesity-diabetes syndromes, there is a marked elevation in muscle and adipose TNF α production, as compared with tissues from lean individuals (Hotamisligil *et al.*, 1995; Hotamisligil *et al.*, 1993). TNF α levels can be reduced with weight loss (Hotamisligil *et al.*, 1995) or after treatment with the insulin-sensitizing agent pioglitazone (Hofmann *et al.*, 1994).

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A third adipocyte product, the acylation stimulating protein (ASP) exert autocrine action on the adipocyte, having potent anabolic effects on human adipose tissue by stimulation of glucose transport and FFA esterification (Maslowska *et al.*, 1997; Walsh *et al.*, 1989). ASP is generated by the interaction of complement D (identical to adipsin), factor B, and complement C3, components of the alternate complement pathway all produced by adipocytes (Choy and Spiegelman, 1996).

White adipose tissue versus brown adipose tissue

There are two different types of adipose tissue in the body, WAT and BAT, which have quite opposite physiological functions although they both have the same “machinery” for lipogenic and lipolytic activity. WAT stores excess energy as triglycerides and releases free fatty acids in response to energy requirements at other sites. BAT on the other hand is involved in adaptive (non-shivering) thermogenesis. BAT is found only at certain sites in the body of rodent, such as in interscapular, perirenal and retroperitoneal regions. In human neonates BAT is present in large quantities but its thermogenic activity decreases shortly after birth and the tissue is gradually converted into white type adipose tissue (Lean *et al.*, 1986). However, judged by expression of the brown fat specific uncoupling protein 1 (UCP1) mRNA, substantial amounts of brown adipocytes exist throughout life in human adipose deposits, which are generally classified as white (Krief *et al.*, 1993).

Brown adipocytes have a multilocular disposition of fat droplets, i.e. a number of individual droplets within each adipocyte, whereas the white adipocyte has a single fat droplet within the cell. Furthermore, the brown adipocyte has a central nucleus and a large number of mitochondria in contrast to the white adipocyte, which has very few mitochondria and a nucleus that is displaced towards the plasma membrane by the lipid droplet. The only known gene marker to distinguish BAT from WAT, or any other cell types is the expression of UCP1 in brown adipocytes. Due to the presence of this unique mitochondrial protein brown adipocytes have the ability of facultative heat production, which is highly regulated by sympathetic nerve activity. UCP1 is a proton translocator in the inner mitochondrial membrane and functions as a

facultative uncoupler of the mitochondrial respiratory chain (Nicholls and Locke, 1984). Recently two new uncoupling proteins have been identified and cloned through their sequence homology with UCP1. UCP2 is found in most tissues (Fleury *et al.*, 1997), while UCP3 is expressed in BAT and skeletal muscle (Boss *et al.*, 1997).

5 The respective roles for UCP2 and UCP3 in thermogenesis and energy balance of intact animals remain to be determined. That brown fat is highly important in rodents for maintaining nutritional homeostasis is predicted by the facts that the function of BAT is impaired in obese rodents (Himms-Hagen, 1989) and transgenic mice with decreased brown fat mass develop obesity (Lowell *et al.*, 1993). Since BAT is much
10 less obvious in large animals like humans, than in rodents, skeletal muscle is thought to be the site of primary importance for normally occurring adaptive thermogenesis in large animals.

Both white and brown fat are innervated under the control of the sympathetic nervous
15 system. There are at least three pharmacologically distinct subtypes of β -adrenergic receptors (β_1 , β_2 , and β_3) found in adipocytes. The β_3 -adrenergic receptor (β_3 -AR) is the predominant subtype in adipose tissue and it mediates the effects of norepinephrine present in the sympathetic synaptic cleft during nerve stimulation of lipolysis in WAT and BAT and of thermogenesis in BAT (Giacobino, 1995).
20 Increased lipolysis takes place primarily through the production of cAMP and the activation of hormone-sensitive lipase through phosphorylation (Fig. 1). Thermogenesis in BAT is accomplished by increased UCP1 mRNA levels through stimulation of transcription (Rehmark *et al.*, 1990; Ricquier *et al.*, 1986). Uncoupled respiration is also thought to be stimulated by increased lipolysis and the raise in
25 intracellular concentration of FFA (Jezek *et al.*, 1994). Sympathetic stimulation of brown fat also contributes to regulation of energy expenditure by increasing mitochondrial biogenesis (Wu *et al.*, 1999a) and hyperplasia of brown adipocytes. In rodents, β_3 -adrenergic receptors (β_3 -ARs) are abundant in WAT and BAT (Granneman *et al.*, 1991; Muzzin *et al.*, 1991; Nahmias *et al.*, 1991), while in
30 humans, β_3 -AR mRNA is abundant in BAT only, with much less or no β_3 -AR mRNA found in WAT (Granneman and Lahners, 1994; Krief *et al.*, 1993). Long-term treatment of obese rodents with β_3 -selective agonists reduces fat stores and improves

obesity-induced insulin resistance (Bloom *et al.*, 1992; Cawthorne *et al.*, 1992; Holloway *et al.*, 1992). Thus, β_3 -selective agonists are promising anti-obesity compounds. Trials of β_3 -AR agonist treatment, aimed at stimulating BAT in humans have proved disappointing with respect to weight loss (Arch and Wilson, 1996). The true potential of β_3 -AR agonists in humans can only be evaluated when a compound with good selectivity and efficacy at the human β_3 -AR, coupled with a long duration of action *in vivo*, has been identified, however those compounds that have been evaluated in humans so far have much lower efficacy at the human than the rodent receptor. This could be explained by the fact that human and mouse/rat β_3 -AR show a ~80% similarity in their amino acid sequence. Several of the β_3 -AR-selective agonists (e.g. BRL 37344 and CL 316,243) have been shown to be extremely potent against mouse and rat β_3 -AR but with a greatly reduced activity against the human β_3 -AR. Presently, recombinant cell lines expressing human β_3 -ARs are being used to identify compounds with an increased potency against the human receptor (Ito *et al.*, 1998). Mice with targeted mutagenesis of the β_3 -AR gene show only a modest tendency to become obese and their brown fat response to cold exposure works perfectly normal (Susulic *et al.*, 1995). Deficient mice displayed an up-regulation of β_1 -AR mRNA levels in both white and brown fat which most probably is the reason of the mild phenotype. These results implicate that it is possible that β_1 - and β_2 -ARs also play important roles in innervation of adipose tissue. Moreover, other species, including humans, have higher levels of β_1 - and β_2 -ARs, then β_3 -AR, in adipose tissue (Lafontan and Berlan, 1993).

Adipocyte differentiation

There has been some great progress during the past few years in the understanding of the adipocyte differentiation program. Most of the work leading to this understanding has been carried out using white preadipose cell lines in culture, notably the C3H10T½ and NIH 3T3 fibroblastic cell lines and the 3T3-L1 and 3T3-F442A preadipocyte cell lines. Treatment of multipotent C3H10T½ cells with 5-azacytidine (a demethylating agent) gives rise to cells committed to the myogenic, adipogenic, osteoblastic, or chondrogenic lineages. This is consistent with the view that the adipose lineage arises from the same multipotent stem cell population of mesodermal origin that gives rise to the muscle and cartilage lineages (Cornelius *et al.*, 1994). Under appropriate hormonal control (e.g. glucocorticoid, insulin-like growth factor-1, and cyclic AMP or factors that mimic these agents) or experimental manipulation white preadipose cell lines are capable to differentiate into mature white adipocytes (Ailhaud *et al.*, 1992). Several transcription factors have been identified, which act co-operatively and sequentially to trigger the functional differentiation program (Fig. 2).

Transcriptional control of adipocyte differentiation through PPARs C/EBPs, and ADD1/SREBP1

Peroxisome proliferator-activated receptors (PPARs) are a class of the nuclear hormone receptors. The member PPARγ is now well recognized as serving an important role in the regulation of adipogenesis. Through the use of alternate promoters, the gene encoding PPARγ gives rise to two separate products, PPARγ1 and PPARγ2, the latter containing an additional 28 N-terminal amino acids that are reported to enhance ligand binding (Fajas *et al.*, 1997; Werman *et al.*, 1997). Reports that ligand activation of retrovirally expressed PPARγ2 in non-differentiating NIH-3T3 cells potently promoted adipocyte differentiation provided the most compelling evidence for the adipogenic nature of PPARγ2 (Tontonoz *et al.*, 1994b). One live-born PPARγ deficient mouse has been produced and it displayed a total absence of

WAT and BAT (complete lipodystrophy) and fatty liver, secondary to lipodystrophy (Barak *et al.*, 1999).

Members of the PPAR family specifically function as heterodimers with the retinoid X receptor (RXR) through interactions with peroxisome proliferator response elements (PPREs) on target genes, including lipoprotein lipase (LPL; Schoonjans *et al.*, 1996), the adipocyte fatty acid-binding protein 422/aP2 (Tontonoz *et al.*, 1994a), phosphoenolpyruvate carboxykinase (PEPCK; Tontonoz *et al.*, 1995), and stearoyl-CoA desaturase 1 (Miller and Ntambi, 1996). Transcriptional activity of PPAR γ is induced following binding of either synthetic or naturally occurring ligands, including prostaglandins of the D2 and J2 series, with the 15-deoxy- Δ 12, 14-prostaglandin J2 derivate emerging as one of the most potent (Forman *et al.*, 1995). Synthetic ligands that activate PPAR γ include carbacyclin and a new class of antidiabetic drugs, the thiazolidinediones (TZDs) (Lehmann *et al.*, 1995). TZDs promote adipogenesis in culture and improve insulin sensitivity *in vivo*. PPAR γ activators probably modify the production of adipocyte-derived mediators of insulin resistance, such as free fatty acids or TNF α . PPAR γ activation will decrease production of TNF α by adipocytes and interfere with its inhibitory effect on insulin signaling (Peraldi *et al.*, 1997).

In addition, because of its tissue selective effects on genes involved in fatty acid uptake, PPAR γ activation will induce repartitioning of fatty acids in the body, with enhanced accumulation of fatty acids in adipose tissue at the expense of a relative depletion of muscle fatty acids (Martin *et al.*, 1997). The relative lipid depletion of muscle cells will improve their glucose metabolism and result in an improvement in insulin sensitivity. Furthermore, PPAR γ decreases the expression of the adipocyte-derived signaling molecule leptin, which results in an increase in energy intake and optimization of energy usage, contributing further to PPAR γ 's adipogenic effect (De Vos *et al.*, 1996). Recently it has been demonstrated that interaction with a novel cofactor PPAR γ coactivator (PGC-1), could enhance PPAR γ transcriptional activity in brown adipose tissue (Puigserver *et al.*, 1998).

Three members of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, *i.e.* C/EBP α , C/EBP β , and C/EBP δ , have been implicated in the induction of adipocyte differentiation. The factors are proteins of the bZIP class, with a basic domain that mediates DNA binding and a leucine zipper dimerization domain.

5 Cyclic AMP and adipogenic hormones such as glucocorticoids and insulin induce a transient increase in the expression of C/EBP β and δ early in adipocyte differentiation (Cao *et al.*, 1991; MacDougald *et al.*, 1994; Yeh *et al.*, 1995). C/EBP β , in synergy with C/EBP δ , then induces PPAR γ expression in the preadipocyte (Wu *et al.*, 1996, Wu *et al.*, 1995). Mice lacking the C/EBP β , and C/EBP δ gene have normal

10 expression of C/EBP α and PPAR γ , but this co-expression of C/EBP α and PPAR γ is not sufficient for complete adipocyte differentiation in the absence of C/EBP β and C/EBP δ (Tanaka *et al.*, 1997). C/EBP α seems to play an important part in the later stages of differentiation by maintaining the differentiated adipocyte phenotype through autoactivation of its own gene (Lin and Lane, 1992; Lin and Lane, 1994).

15 C/EBP α activates several adipocyte-specific genes such as the insulin-responsive glucose transporter-4 (GLUT4) (Kaestner *et al.*, 1990), 422/aP2 (Christy *et al.*, 1989), UCP1 (Yubero *et al.*, 1994), and also the insulin receptor gene, and insulin receptor substrate 1 (IRS-1) (Wu *et al.*, 1999b). Definitive proof that C/EBP α is required for adipocyte differentiation was obtained by showing that expression of antisense

20 C/EBP α RNA in 3T3-L1 preadipocytes prevented differentiation (Samuelsson *et al.*, 1991). Consistent with this finding, disruption of the C/EBP α gene gave rise to mice that failed to develop white adipose tissue (Wang *et al.*, 1995). Taken together these findings proved that C/EBP α is both required and sufficient to induce adipocyte differentiation. The expression of C/EBP α , as well as other adipocyte genes, is

25 induced upon ligand activation of PPAR γ . Through a positive feedback loop, C/EBP α maintains the expression of PPAR γ . C/EBP α and PPAR γ cooperate to promote adipocyte differentiation, including adipocyte gene expression and insulin sensitivity (Wu *et al.*, 1999b). It is possible that C/EBP α is ultimately an important, indirect target of the antidiabetic actions of the TZDs.

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ADD1/SREBP1 (adipocyte determination and differentiation-dependent factor 1/sterol regulatory element binding protein 1) is a member of the basic helix-loop-helix (bHLH) class of transcription factors. In the inactive state, the protein is membrane-bound to the endoplasmic reticulum. Upon activation (such as a low cholesterol state), ADD1/SREBP1 is proteolytically cleaved and the soluble form becomes translocated to the nucleus where it binds one of two different response elements, namely the E box and the sterol regulatory element (SRE; Brown and Goldstein, 1997). The expression of ADD1/SREBP1 is induced during differentiation of adipocytes, where it activates transcription of target genes involved in both cholesterol metabolism and fatty acid metabolism (Kim and Spiegelman, 1996). ADD1/SREBP1 potentiates the transcriptional activity of PPAR γ probably through the production of endogenous ligands for PPAR γ (Kim *et al.*, 1998) and also by binding to and inducing the PPAR γ promoter (Fajas *et al.*, 1999).

When preadipocytes differentiate into adipocytes, several differentiation-linked genes are activated. Lipoprotein lipase (LPL) is one of the first genes induced during this process (Fig. 2). Two cis-regulatory elements important for gradual activation of the LPL gene during adipocyte development *in vitro* have been delimited (Enerback *et al.*, 1992). These elements, LP- α and LP- β , contained a striking similarity to a consensus sequence known to bind transcription factors of the winged helix family. Results of gel mobility shift assays and DNase I and exonuclease III *in vitro* protection assays indicated that factors with DNA-binding properties similar to those of the winged helix family of transcription factors are present in adipocytes and interact with LP- α and LP- β . There is a need for identifying human winged helix genes that could be responsible for the induction of the LPL promoter and possibly regulating expression of other adipocyte specific genes.

“Fork head” and “winged helix” genes

The “fork head” domain is an evolutionary conserved DNA-binding domain of 100 amino acids, which emerged from a sequence comparison of the transcription factor HNF-3 α of rat and the homeotic gene *fork head* of *Drosophila*. X-ray crystallography

of the fork head domain from HNF-3 γ revealed a three-dimensional structure, the “winged helix”, in which two loops (wings) are connected on the C-terminal side of the helix-turn-helix (Brennan, R.G. (1993) Cell 74, 773-776; Lai, E. et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10421-10423).

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The isolation of the mouse mesenchyme fork head-1 (MFH-1) and the corresponding human (*FKHL14*) chromosomal genes is disclosed by Miura, N. et al. (1997) Genomics 41, 489-492. The nucleotide sequences of the mouse MFH-1 gene and the human *FKHL14* gene have been deposited with the EMBL/GenBank Data Libraries under accession Nos. Y08222 and Y08223 (SEQ ID NO: 1), respectively. The International Patent Application WO 98/54216 (published on December 3, 1998) discloses a gene designated *freac11*, which encodes a polypeptide identical to polypeptide encoded by the human *FKHL14* gene disclosed by Miura.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1

Schematic view of lipogenic and lipolytic actions in the adipocyte. Triglycerides are hydrolysed to glycerol and free fatty acids by the action of lipoprotein lipase. FFAs are transported into the adipocyte via FATP. FFAs are combined with acetyl CoA to produce acyl CoA, which is re-esterified to triglycerides. The multi-enzyme complex hormone sensitive lipase hydrolyses triglycerides into FFAs and glycerol. FFAs can either be re-esterified again or be released into the circulation. LPL, lipoprotein lipase; FFA, free fatty acid; FATP, fatty acid transport protein; aP2, adipocyte fatty acid-binding protein 422/aP2; ACS, acyl CoA synthetase; Glut4, glucose transporter IV; β AR, β -adrenergic receptor; AC, adenylate cyclase; PKA, protein kinase A; HSL, hormone sensitive lipase. Figure is adapted from Sethi and Hotamisligil, 1999.

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FIGURE 2

Summary of different stages and events during *in vitro* adipocyte differentiation. Our current understanding of adipocyte differentiation indicates that a pluripotent stem

cell precursor gives rise to a mesenchymal precursor cell (multipotent) with the potential to differentiate along mesodermal lineages of myoblast, chondroblast, osteoblast, and adipocyte. Given appropriate environmental and gene expression cues, preadipocytes undergo clonal expansion and subsequent terminal differentiation.

5 Selected molecular events accompanying this process are indicated above. Pref-1, preadipocyte factor 1; pOb24/A2COL6, $\alpha 2$ chain of type VI collagen; LPL, lipoprotein lipase; aP2, adipocyte fatty acid-binding protein 422/aP2; UCP, uncoupling protein; CUP, C/EBP α undifferentiated protein; FAAR, fatty acid-activated receptor; PPAR γ , peroxisome proliferator-activated receptor γ ; C/EBP,

10 CCAAT/enhancer binding protein; RXR, retinoid X receptor. Figure is adapted from Klaus, 1997.

FIGURE 3

Northern blot analysis of adult human tissues (A) and adult mouse tissues (B). The

15 blots were analyzed with probes specific for *FKHL14/FOXC2* (human) and *Mfh1* (mouse) respectively. The experiments were carried out with 20 μ g of total RNA/lane and β -actin was used as a control.

FIGURE 4

20 CHO cells transiently transfected with Ob reporter- and *FKHL14/FOXC2* expression plasmids. The Ob promoter activity was determined by a luciferase activity assay. Data are means \pm SD from two independent experiments.

FIGURE 5

25 Schematic view of *FKHL14/FOXC2* transgene construct and Southern blot detection of integration positive mice. A 2.1-kb fragment containing the *FKHL14/FOXC2* cDNA was ligated downstream of the 5.4-kb aP2 enhancer/promoter region (A). Prior to pronuclear injection the transgene construct was liberated from the plasmid as an 8.9-kb *NotI/AgeI* fragment. A 1.7-kb fragment of the aP2 promoter was used as a

30 probe in Southern blot analysis for identification of integration positive mice. Panel B shows the identification of the three founders investigated further.

FIGURE 6

Expression levels of the *FKHL14/FOXC2* transgene in white adipose tissue (WAT) and brown adipose tissue (BAT) of wild-type mice (WT) and *FKHL14/FOXC2* transgenic mice (founder A, B, and C) as measured by Northern blot analysis (12 µg of total RNA/lane). GAPDH was used as a control.

FIGURE 7

A 5-months-old wild-type female and a *FKHL14/FOXC2* transgenic female littermate. A, C, and E show WT; B, D, and F show transgenic littermate. (A, B) An exposed dorsal view of the interscapular brown fat pads, illustrating an increased size of the depot in the transgenic mouse. (C, D) An exposed ventral view, illustrating a reduction in size and change in appearance of the intrabdominal white fat pads in the transgenic mouse. (E, F) The interscapular brown fat pads and the intrabdominal white fat pads have been dissected out. BAT at the top and WAT at the bottom.

FIGURE 8

Histologic sections of brown fat (A, B), and white fat (C, D) from a 5-months-old wild-type mouse (left) and a *FKHL14/FOXC2* transgenic littermate (founder A) (right). (A, B) Interscapular brown fat of the transgenic mouse consists of markedly enlarged adipocytes containing large unilocular fat droplets. (C, D) Adipocytes in white fat of the transgenic mouse show heterogeneity of size. This is in contrast to the WAT from a wild-type mouse, which consists of adipocytes of uniform size filled with a large, unilocular vacuole.

FIGURE 9

Change of size of fat depots in *FKHL14/FOXC2* transgenic mice (founder C). (A) Weight comparison (expressed as percent of total body weight) for intrabdominal fat depots and interscapular brown fat depots of 5-months-old wild-type females and *FKHL14/FOXC2* transgenic littermates. Changes are significant, $P < 0.005$. (B) Ratio between weights of the intrabdominal fat depot and the interscapular brown fat depot. Change is significant, $P < 0.0005$. (C) No significant difference in body weight could be detected between the two groups. (D) No significant difference in food

consumption was noticed when measured during a time period of two months. Data are means \pm SD, n=3 for both WT and founder C in A-C, n=4 for both WT and founder C in D.

5 FIGURE 10

Amount of different mRNAs in white adipose tissue (WAT) and brown adipose tissue (BAT) of wild-type mice (WT) and *FKHL14/FOXC2* transgenic mice (founder A, B, and C) as measured by Northern blot analysis (12 μ g of total RNA/lane). GAPDH was used as a control for ensuring equal loading on all blots.

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FIGURE 11

Serum triglyceride is lowered in *FKHL14/FOXC2* transgenic mice (founder A). Serum triglyceride content was analyzed for 14-weeks-old wild-type males and *FKHL14/FOXC2* transgenic littermates fed ad libitum. Change is significant, P<0.005. Data are means \pm SD, n=4 for both WT and founder A.

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FIGURE 12

Total body lipid content is lowered in *FKHL14/FOXC2* transgenic mice (founder A). Total body lipid content was analyzed for 6-months-old wild-type males and *FKHL14/FOXC2* transgenic littermates fed ad libitum. Change is significant, P<0.0005. Data are means \pm SD; WT, n=3 and founder A, n=4.

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FIGURE 13

Blood glucose is lowered (A) and glucose elimination is more efficient (B) in *FKHL14/FOXC2* transgenic mice (founder A). (A) Blood glucose levels were analysed for 10-weeks-old wild-type mice and *FKHL14/FOXC2* transgenic littermates fed ad libitum. A significant (P<0.05) reduction of blood glucose levels were seen for *FKHL14/FOXC2* transgenic mice. (B) An intravenous glucose tolerance test was carried out on the mice used in (A). Blood samples were taken immediately before and at 1, 5, 20, and 50 min after intravenous injection of glucose (1 g/kg). The values were significantly changed at 0 min (P<0.05), 20 min (P<0.001), and at 50 min (P<0.05). Data are means \pm SD; n=10 for both WT and founder A.

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FIGURE 14

Plasma insulin is lowered in *FKHL14/FOXC2* transgenic mice (A), still levels raised higher than in wild-type after intravenous (iv) glucose load (B, C) (founder A). (A) Plasma insulin levels were analyzed for 10-weeks-old wild-type mice and *FKHL14/FOXC2* transgenic littermates fed ad libitum. A significant ($P<0.05$) reduction of plasma insulin levels was seen for *FKHL14/FOXC2* transgenic mice. (B) An intravenous glucose tolerance test was carried out on the mice used in (A) Blood samples were taken immediately before and at 1, 5, 20, and 50 min after intravenous injection of glucose (1 g/kg). The values were significantly changed at 0 min ($P<0.05$), 20 min ($P<0.01$), and at 50 min ($P<0.05$). (C) Fold induction of plasma insulin levels one minute after i.v. glucose load. Change is significant, $P<0.005$. Data are means \pm SD; $n=10$ for both WT and founder A.

FIGURE 15

Hypothetical action of *FKHL14/FOXC2* in adipocytes. Filled arrow indicates known positive transcriptional regulation. Open arrow represents a proposed action of *FKHL14*. ADD1/SREBP1 both activates transcription of acetyl CoA carboxylase (Lopez et al., 1996), fatty acid synthase (FAS), and lipoprotein lipase (LPL), and increases the transcriptional activity of PPAR γ (Fajas et al., 1999). PPAR γ activates transcription of fatty acid transport protein (FATP), acyl-CoA synthetase genes (ACS; Martin et al., 1997), adipocyte fatty acid-binding protein 422/aP2 (aP2; Tontonoz et al., 1994a), LPL (Schoonjans et al., 1996), and phosphoenolpyruvate carboxykinase (PEPCK; Tontonoz et al., 1995). C/EBP α activates insulin-responsive glucose transporter-4 (GLUT4; Kaestner et al., 1990), aP2 (Christy et al., 1989), uncoupling protein 1 (UCP1; Yubero et al., 1994), the insulin receptor (InsR), insulin receptor substrate-1 (IRS-1; Wu et al., 1999b), and PEPCK (Park et al., 1990). A positive feedback loop between C/EBP α and PPAR γ have been suggested (Wu et al., 1999b).

FIGURE 16

Reduction in weight gain in (transgenic) tg mice. Analysis were performed on tg-A mice with wt littermates as controls, fed *ad libitum*, mice were approximately 4-6 months of age. There is a reduction in diet induced weight gains in

*FKHL14/FOXC2*tg mice, both in females ($p<0.02$; **a**) and males ($p<0.03$; **b**), as compared with wt mice, values are means \pm SEM, $n=4$ in each group. Mice were on a high fat diet (58.0% on a caloric basis) for seven weeks (see Methods).

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DISCLOSURE OF THE INVENTION

According to the present invention, the human transcription factor gene *FKHL14/FOXC2* is identified as a key regulator of adipocyte metabolism. Increased *FKHL14/FOXC2* expression, in white (WAT) and brown adipose tissue (BAT), has a pleiotropic effect on gene expression, which leads to resistance to diet induced weight gain and a decrease in: total body lipid content, serum triglycerides, plasma levels of free fatty acids, glucose and insulin. To our knowledge, *FKHL14/FOXC2* is the hitherto only identified gene that, in a concerted action, can counteract most, if not all, of the symptoms associated with obesity, including hypertriglyceridemia and insulin resistance – a likely consequence hereof would be protection against type 2 diabetes.

During adulthood, the human winged helix gene *FKHL14/FOXC2* is expressed exclusively in adipose tissue. The LPL mRNA levels in the *FKHL14/FOXC2* transgenic mice seem to be slightly elevated (Fig. 10), which is in concordance with the initial findings that the two winged helix cis-regulatory elements are responsible for the inducibility of the LPL promoter (Enerback *et al.*, 1992). The higher expression level of LPL most probably is responsible for the significantly decreased plasma TG levels noticed in *FKHL14/FOXC2* transgenic mice (Fig. 11), in addition to the fact that the profound up-regulation of adipsin in both WAT and BAT (Fig. 10) most certainly is of great importance. Adipsin is a secreted protein necessary for the formation of acylation stimulating protein (ASP), which has potent anabolic effects on human adipose tissue for both glucose and free fatty acid (FFA) storage (Cianflone *et al.*, 1995).

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The extensive alternations seen in the *FKHL14/FOXC2* transgenic mice presented here predicts a very central and important role for this winged helix gene hitherto

unknown to participate in molecular events in adipose tissue. It has been demonstrated by gene targeting experiments that the mouse homologue, *Mfh1*, plays a crucial role during embryonic development (Iida *et al.*, 1997; Winnier *et al.*, 1997), but so far nothing has been published about its function in adult mice. In a recent publication an *Mfh1/Pax1* double mutant was shown to be totally absent of BAT at day 15.5 dpc (Furumoto *et al.*, 1999).

The *FKHL14/FOXC2* transgenic mice had a clear reduction in white adipose tissue mass. The reduced size of the white fat depots might be due solely to the reduction in size of the adipocytes (Fig. 8), but one cannot rule out the possibility that there is also a reduction in adipocyte number. There are a number of possible reasons why the *FKHL14/FOXC2* transgenic mice have decreased size of white adipocytes. One might presume an increased lipolytic activity of their adipocytes in general; coupled with the greater mass of brown fat this may lead to increased energy expenditure through heat production by BAT giving rise to the lean phenotype. The upregulation of β_3 -AR seen in transgenic WAT (Fig. 10) will result in an elevated activation of HSL hence an increase in lipolysis (Fig. 1) ultimately leading to reduced lipid storing.

Furthermore, the transgenic mice were insulin sensitive (Fig. 14); this could be explained by both the upregulation of genes involved in insulin action (InsR, IRS-1, IRS-2, and GLUT4; Fig. 10) and the lean body composition (Fig. 12). Insulin has a critical role in lipid metabolism, promoting the storage of triglycerides in adipocytes through numerous actions on this cell. Among these are stimulation of glucose uptake and inhibition of lipolysis, which occur very rapidly through insulin-responsive glucose transporter protein (GLUT 4) translocation or covalent modification of HSL, respectively. Insulin also stimulates fatty acid and triglyceride synthesis, through the induction of key lipogenic enzymes and induction of lipoprotein lipase. The data presented here are compatible with an increased energy turnover in the adipocytes derived from *FKHL14/FOXC2* transgenic mice.

The white fat depots of *FKHL14/FOXC2* transgenic mice had an ectopic expression of the brown fat specific marker UCP1 (Fig. 10). The origin of multilocular adipocytes in transgenic WAT (Fig. 8d) remains an enigma. It has been suggested that

there is a pool of intraconvertible cells or small brown preadipocytes present in WAT. Studies on both rat and mice have demonstrated atypical occurrence of UCP1 in certain WAT depots previously thought to contain only white adipocytes (Cousin *et al.*, 1992; Loncar, 1991). If submitted to cold or to treatment with a β_3 -AR selective agonist, UCP1 expression was increased in WAT as in typical BAT and on histological sections one could identify small multilocular cells interspersed between the white adipocytes, which were shown to contain UCP1 by immunohistochemistry (Cousin *et al.*, 1992; Ghorbani *et al.*, 1997). Furthermore, both UCP1 and β_3 -AR mRNAs have been detected in white fat depots of human beings (Krief *et al.*, 1993) and recently, it has been shown that cultures of human adipocytes derived from white fat depots express UCP1 after treatment with β_3 -AR agonists (Champigny and Ricquier, 1996). Moreover, transgenic mice overexpressing β_1 -AR in WAT and BAT have abundant appearance of brown fat cells in subcutaneous WAT (Soloveva *et al.*, 1997). We would like to speculate that these suggested intraconvertible cells or small brown preadipocytes have undergone proliferation in our transgenic mice, readily detectable on histological sections as small multilocular cells (Fig. 8d), due to the increased expression of β_3 -AR. The *FKHL14/FOXC2* transgene possibly activates other proteins further down the signal transduction pathway finally leading to the induction of UCP1 expression. In addition, the levels of C/EBP α and PPAR γ mRNAs in transgenic WAT reaches the ones in wild-type BAT (Fig. 10) and both of these transcription factors are known to induce UCP1 expression (Digby *et al.*, 1998; Yubero *et al.*, 1994). C/EBP α activates several adipocyte-specific genes and also genes involved in insulin action (Fig. 15), hence C/EBP α (-/-) cells show a complete absence of insulin-stimulated glucose transport, secondary to reduced gene expression and tyrosine phosphorylation for the insulin receptor and IRS-1 (Wu *et al.*, 1999b). The WAT of *FKHL14/FOXC2* transgenic mice have marked elevation of the mRNA levels for both C/EBP α and PPAR γ , these transcription factors may in turn be responsible for upregulation of mRNA levels for aP2, LPL, UCP1, GLUT4, insulin receptor, and IRS-1 (Fig. 15)

It is interesting to note that the white adipocytes of *FKHL14/FOXC2* transgenic mice have not just converted into brown adipocytes, in the meaning of mRNA expression,

as they have for example higher levels of certain mRNAs (i.e. β_2 -AR, insulin receptor, IRS-1, and IRS-2) than seen in any type of wild-type adipose tissue.

In the mice studied here, *FKHL14/FOXC2* transgene expression was under the control of the aP2 promoter, which only functions in adipocytes and not in stem cells and probably neither in intraconvertible cells discussed above (Ailhaud *et al.*, 1992). Considering the fact that aP2 is a late marker (Fig. 2) it is quite surprising that we obtain such a dramatic change in the characteristics of white adipocytes. Currently, it is not known if adipocyte dedifferentiation occurs *in vivo*, whereas it has been demonstrated *in vitro* that this process occurs and is induced by $\text{TNF}\alpha$ in human adipocytes (Petruschke and Hauner, 1993). Another interpretation for the occurrence of small multilocular cells in WAT of our transgenic mice could then be a dedifferentiation of originally white adipocytes followed by a conversion into the type of adipocytes observed in the *FKHL14/FOXC2* transgenic mice.

The interscapular brown fat of *FKHL14/FOXC2* transgenic mice weighed ~7.5 times as much as wild-type brown fat (Fig. 9a). This extreme hypertrophy might be explained by the increased expression of β_3 - and β_2 -AR mRNA seen in BAT of *FKHL14/FOXC2* transgenic mice (Fig. 10). Chronic treatment with β_3 -AR agonists increases body temperature and energy expenditure and it causes hypertrophy of the interscapular BAT, with several fold increases in the content of UCP1 and cytochrome oxidase (Himms-Hagen *et al.*, 1994). The morphology of transgenic interscapular BAT is somewhat changed having larger fat droplets than wild-type BAT (Fig. 8a & b). This is not a feature of chronic β_3 -AR agonist treatment, but there is a possibility that the upregulation of markers involved in insulin action (Fig. 10) and elevated levels of adiponin promotes the increased storage of triglycerides in brown adipocytes of transgenic mice. It has also been noticed before that elevated levels of UCP2 mRNA can be coupled to this phenotype (Enerback *et al.*, 1997; Kozak *et al.*, 1991).

Dysfunctional BAT seen in the ADD1/nSREBP-1c transgene (Shimomura *et al.*, 1998) and genetically ablated BAT in the UCP1-DTA transgene (UCP1 promoter –

diphtheria toxin A chain) (Lowell *et al.*, 1993) leads to insulin resistance. Transgenic mice overexpressing ADD1/nSREBP-1c displays several features quiet opposite the ones of the *FKHL14/FOXC2* transgene, including insulin resistance and NIDDM. *FKHL14/FOXC2* transgenic mice displays a somewhat opposite change of expression pattern compared with that of ADD1/nSREBP-1c transgenic mice, there the mRNAs encoding PPAR γ , C/EBP α , aP2, UCP1, adipsin, InsR, IRS-1, IRS-2, and GLUT4 all are downregulated. In our transgene all of this mRNAs are instead upregulated (Fig. 10). However, intriguingly our transgenic mice actually have raised levels of ADD1/SREBP1 mRNA in WAT (Fig. 10) somewhat mimicking the ADD1/nSREBP-1c transgene in that regard.

FKHL14/FOXC2 might be an important participant in the regulation of leptin expression, based on the fact that leptin mRNA levels are down-regulated in *FKHL14/FOXC2* transgenic mice (most prominent in BAT; Fig. 10), and the ten-fold decrease of Ob promoter activity seen in cell culture experiments then cotransfected with *FKHL14/FOXC2* expression plasmid (Fig. 4). In addition, leptin expression is inhibited by β_3 -adrenergic stimuli (Mantzoros *et al.*, 1996), which is presumed to be high in *FKHL14/FOXC2* transgenic mice due to the up-regulation of β_3 -AR mRNA levels.

The amount of food consumed by transgenic mice compared to that of wild-type did not differ (Fig. 9d), and no significant difference in body weight has been observed, predicting that the difference observed in total body lipid content must be compensated with an increased anabolism in *FKHL14/FOXC2* transgenic mice. The *FKHL14/FOXC2* transgenic mice most probably also are protected against developing diet-induced obesity, taking in consideration the observed insulin sensitivity (Fig. 14), the lower blood glucose levels and more efficient glucose elimination (Fig. 13) observed in our transgenic mice. Insulin sensitivity and/or resistance to diet-induced obesity have been observed for several other transgenic mouse models: targeted disruption of the RII β subunit of protein kinase A results in lean mice resistant to diet-induced obesity (Cummings *et al.*, 1996), mice lacking the protein tyrosine phosphatase-1B gene (PTP-1B) are insulin sensitive and resistant to obesity (Elchebly

et al., 1999), aP2-UCP1 transgenic mice are prevented against genetic obesity (Kopecky *et al.*, 1995), and transgenic mice overexpressing the β_1 -AR in adipose tissue are resistant to obesity (Soloveva *et al.*, 1997). Moreover, β_3 -AR agonists have been found to have anti-diabetic effects in animal models of obesity and NIDDM; chronic dosing can improve glucose tolerance, increase insulin sensitivity and reduce fasting blood glucose levels (Cawthorne *et al.*, 1992). *FKHL14/FOXC2* is the only adipocyte specific gene that, directly or indirectly, regulates triglyceride metabolism, adrenergic regulation and insulin action in adipocytes. Actually, the *FKHL14*, is to our knowledge the only known gene that, in a concerted action, can counteract most, if not all, of the symptoms associated with obesity: hypertriglyceridemia, insulin resistance and most likely the associated clinical syndrome of NIDDM.

The apparent *FKHL14/FOXC2* transgene dose responsive effect observed in WAT for the induction of UCP1, β_3 -AR, and adipsin, may indicate a direct interaction for *FKHL14/FOXC2* with the promoters of these genes. A schematic view of the hypothetical action of *FKHL14/FOXC2* in adipocytes is shown in Fig. 15.

According to the present invention, proper activation of *FKHL14/FOXC2* by drugs may decrease fat stores, while preserving skeletal muscle mass, by preventing fat assimilation during digestion and by increasing WAT lipolysis, BAT thermogenesis, and insulin action. Such drugs may thus prove useful in treating obesity and NIDDM as well as associated diseases. It is thus foreseen that an effective amount of a polypeptide encoded by the human *FKHL14/FOXC2* gene, could be useful in methods for the treatment of medical conditions related to obesity.

In another aspect, this invention relates to a construct, or more specifically a gene construct or recombinant construct, comprising a human *FKHL14/FOXC2* nucleotide sequence operably linked to an element selected from the group consisting of promoters, response elements, enhancer elements and mixtures thereof. The term “operably linked” as used herein means functionally fusing an element with a structural gene in the proper frame to express the structural gene under control of the element.

Preferably, the said element is a promoter, in particular an adipose-specific promoter such as the adipose-specific promoter of the murine gene encoding adipocyte P2 (Fig. 5), which can be isolated as described by Ross et al. (1990).

5

In a preferred form of the invention, the said *FKHL14/FOXC2* nucleotide sequence is identical or substantially similar with SEQ ID NO: 1 of the Sequence Listing. However, the *FKHL14/FOXC2* nucleotide sequence is not to be limited strictly to the sequence shown as SEQ ID NO: 1. Rather the invention encompasses constructs comprising nucleotide sequences carrying modifications like substitutions, small deletions, insertions or inversions, which nevertheless encode polypeptides having substantially the biochemical activity of the FKHL14/FOXC2 polypeptide.

10

Consequently, included in the invention are constructs wherein the said human *FKHL14/FOXC2* nucleotide sequence is selected from:

15

(a) the nucleotide sequence shown as SEQ ID NO: 1;
(b) nucleotide sequences capable of hybridizing, under stringent hybridization conditions, to a nucleotide sequence complementary to the polypeptide coding region of a nucleotide sequence as defined in (a) and which codes for a biologically active FKHL14/FOXC2 polypeptide, or a functionally equivalent modified form thereof;
(c) nucleic acid sequence which are degenerate as a result of the genetic code to a nucleotide sequence as defined in (a) or (b) and which codes for a biologically active FKHL14/FOXC2 polypeptide, or a functionally equivalent modified form thereof;
and

20

(d) nucleotide sequences which are at least 90% homologous, preferably at least 95% homologous, with the nucleotide sequence shown as SEQ ID NO: 1 in the Sequence Listing.

25

The term "stringent hybridization conditions" is known in the art from standard protocols (e.g. Ausubel et al) and could be understood as e.g. hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at +65°C, and washing in 0.1xSSC / 0.1% SDS at +68°C.

30

In another aspect, the invention provides a transgenic non-human mammalian animal whose genome comprises a gene construct as defined above, said animal being capable of expressing the human *FKHL14/FOXC2* gene in its adipose tissue.

5 By “transgenic animal” is meant a non-human mammalian animal that includes a nucleic acid sequence which is inserted into a cell and becomes a part of the genome of the animal that develops from that cell. Such a transgene may be partly or entirely heterologous to the transgenic animal. Although transgenic mice represent a preferred embodiment of the invention, other transgenic mammals, including transgenic rodents (for example, hamsters, guinea pigs, rabbits, and rats), and transgenic pigs, cattle, 10 sheep, and goats may be constructed by standard techniques and are included in the invention.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected 15 out of their oviducts. The eggs are stored in an appropriate medium. DNA or cDNA encoding the *FKHL14/FOXC2* gene is purified from a vector by methods well known in the art. Tissue specific regulatory elements, such as the adipocyte-specific aP2 promoter discussed above, may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, 20 is put into a microinjection needle and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse, where it proceeds to the uterus, implants, and develops to term.

25 A transgenic mouse according to the invention could preferably be derived from a genetically obese mouse. Genetically obese mice, such as ob/ob or db/db mice, are well known in the art.

30 In a further aspect, the invention provides an isolated cell line derived from the transgenic non-human mammalian animal. In yet another aspect, the invention provides a method for producing a transgenic non-human mammalian animal overexpressing the human *FKHL14/FOXC2* gene, said method comprising

chromosomally incorporating a gene construct comprising the human *FKHL14/FOXC2* gene, together with suitable regulatory sequences, into the genome of said non-human mammalian animal.

5 The invention also provides a method for studying the biological activity of a polypeptide encoded by the human *FKHL14/FOXC2* gene, said method comprising the steps (i) producing a transgenic non-human mammalian animal overexpressing the human *FKHL14/FOXC2* gene; and (ii) comparing the phenotype of the said transgenic non-human mammalian animal with a wild-type animal of the same
10 species.

In further important aspects, the invention provides biological screening assays for the identification of compounds that could be useful for the treatment of medical conditions related to obesity, or alternatively, to malnutrition. A “medical condition
15 related to obesity” includes e.g. obesity, NIDDM, hypertension and hyperlipidemia. The said “medical condition related to malnutrition” includes e.g. anorexia, ineffective metabolism, and cancer.

Consequently, the invention provides a method for identifying a compound useful for
20 the treatment of a medical condition related to obesity, said method comprising the steps (i) contacting a test compound with the human *FKHL14/FOXC2* gene; and (ii) determining whether said test compound activates the expression of the human *FKHL14/FOXC2* gene, such activation being indicative for a compound useful for the treatment of a medical condition related to obesity.

25 In another aspect, the invention provides a method of screening for a compound useful for the treatment of a medical condition related to obesity, said method comprising exposing a non-human mammalian animal to a test compound, and determining the activity of said human *FKHL14/FOXC2* gene in said non-human
30 mammalian animal, wherein an increase in said gene activity as compared to an untreated non-human mammalian animal being indicative of a compound useful for the treatment of a medical condition related to obesity.

The said non-human mammalian animal is preferably a mouse, for example an obese mouse. Mice can be rendered obese by administration of a high-fat diet. Alternatively, the mouse can be a genetically obese mouse, such as an ob/ob or db/db mouse.

5 In the methods described above, the activity of the human *FKHL14/FOXC2* gene in said non-human mammalian animal, can advantageously be compared to the activity of the said human *FKHL14/FOXC2* gene in a transgenic non-human mammalian animal expressing or overexpressing the said human *FKHL14/FOXC2* gene.

10 In an alternative method for identifying a compound useful for the treatment of a medical condition related to obesity, the method comprises the steps (i) contacting a test compound with a polypeptide encoded by the human *FKHL14/FOXC2* gene; and (ii) determining whether said test compound stimulates the biological activities of the said polypeptide, such stimulation being indicative for a compound useful for the
15 treatment of a medical condition related to obesity. The term “biological activities”, as used in this context, means e.g. enhancing the DNA-protein interaction between the *FKHL14/FOXC2* polypeptide and target sequences in promoters of target genes.

The invention further provides a method for identifying a compound useful for the
20 treatment of a medical condition related to malnutrition, said method comprising the steps (i) contacting a test compound with the human *FKHL14/FOXC2* gene; and (ii) determining whether said test compound decreases or inhibits expression of the *FKHL14/FOXC2* gene, such decrease or inhibition being indicative for a compound useful for the treatment of a medical condition related to malnutrition.

25 Also included in the invention is a method of screening for a compound useful for the treatment of a medical condition related to malnutrition, said method comprising exposing a non-human mammalian animal, preferably a mouse, such as an obese mouse, to a test compound, and determining the activity of said human
30 *FKHL14/FOXC2* gene in said non-human mammalian animal, wherein a decrease in said gene activity as compared to an untreated non-human mammalian animal being

indicative of a compound useful for the treatment of a medical condition related to malnutrition.

5 In an alternative method for identifying a compound useful for the treatment of a medical condition related to malnutrition, the method comprises the steps (i) contacting a test compound with a polypeptide encoded by the human *FKHL14/FOXC2* gene; and (ii) determining whether said test compound decreases or inhibits the biological activities of the said polypeptide, such decrease or inhibition being indicative for a compound useful for the treatment of a medical condition
10 related to malnutrition.

For therapeutic uses, the compositions or agents identified using the methods disclosed herein may be administered systemically, for example, formulated in a pharmaceutically acceptable buffer such as physiological saline. Preferable routes of
15 administration include, for example, oral, subcutaneous, intravenous, intraperitoneally, intramuscular, or intradermal injections, which provide continuous, sustained levels of the drug in the patient. Treatment of human patients or other animals will be carried out using a therapeutically effective amount of an identified compound in a physiologically acceptable carrier. Suitable carriers and their
20 formulation are described, for example, in Remington's Pharmaceutical Sciences by E. W. Martin. The amount of the active compound to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the type of disease and extensiveness of the disease. Generally, amounts will be in the range of those used for other agents used in the treatment of obesity and
25 diabetes.

In a further important aspect of the invention, the human *FKHL14/FOXC2* gene could be used in gene therapy of medical conditions related to obesity. Included in the invention is thus a method of treating obesity in a human, comprising (i)
30 administering to the said human a vector comprising a human *FKHL14/FOXC2* DNA sequence operably linked to a promoter; and (ii) allowing the said human to express a therapeutically effective amount of a polypeptide encoded by said human

FKHL14/FOXC2 gene. A gene delivery system including said vector, comprising a human *FKHL14/FOXC2* DNA sequence operably linked to a promoter, is in itself another aspect of the invention.

The *FKHL14/FOXC2* gene should be operably linked to at least one element which allows for expression of the gene when introduced into the host cell environment. These sequences include promoters, response elements, and enhancer elements. Preferred is the adipose-specific promoter/enhancer of the murine gene encoding adipocyte P2

The heterologous gene may be delivered to the organism using a vector or other delivery vehicle. DNA delivery vehicles can include viral vectors such as adenoviruses, adeno-associated viruses, and retroviral vectors. See, for example: Chu et al. (1994) *Gene Ther* 1: 292-299; Couture et al. (1994) *Hum Gene Ther* 5:667-677; and Eiverhand et al. (1995) *Gene Ther* 2: 336-343. Non-viral vectors which are also suitable include DNA-lipid complexes, for example liposome-mediated or ligand/poly-L-Lysine conjugates, such as asialoglyco-protein-mediated delivery systems. See, for example: Feigner et al. (1994) *J. Biol. Chem*, 269: 2550-2561; Derossi et al. (1995) *Restor. Neurol. Neuros.* 8: 7-10; and Abcallah et al. (1995) *Biol. Cell* 85: 1-7.

If a vector is chosen as the delivery vehicle for the gene, it may be any vector which allows expression of the gene in the host cells. It is preferable if the vector also is one that is capable of integrating into the host genome, so that the gene can be expressed permanently. Ad (adenovirus) vectors have been exploited for the delivery of foreign genes to cells for a number of reasons, including the fact that Ad vectors have been shown to be highly effective for the transfer of genes into a wide variety of tissues *in vivo* and the fact that Ad infects both dividing and non-dividing cells. The vector is administered to the host, generally by intravenous injection. Suitable titers will depend on a number of factors, such as the particular vector chosen, the host, strength of promoter used and the severity of the disease being treated.

Alternatively, it is contemplated that in some human disease states, preventing the expression of, or decreasing the activity of, the human FKHL14/FOXC2 gene will be useful in treating disease states. It is contemplated that antisense therapy or gene therapy could be applied to negatively regulate the expression of the human FKHL14/FOXC2 gene. Antisense nucleic acids (preferably 10 to 20 base-pair oligonucleotides) capable of specifically binding to FKHL14/FOXC2 expression control sequences or FKHL14/FOXC2 RNA are introduced into cells (e.g. by a viral vector or colloidal dispersion system such as a liposome). The antisense nucleic acid binds to the FKHL14/FOXC2 target nucleotide sequence in the cell and prevents transcription and/or translation of the target sequence. Phosphorothioate and methylphosphonate antisense oligonucleotides are specifically contemplated for therapeutic use by the invention. The antisense oligonucleotides may be further modified by poly-L-lysine, transferrin polylysine, or cholesterol moieties at their 5'-end. Suppression of FKHL14/FOXC2 expression at either the transcriptional or translational level is useful to generate cellular or animal models for diseases/conditions characterized by aberrant FKHL14/FOXC2 expression.

Throughout this description the terms "standard protocols" and "standard procedures", when used in the context of molecular biology techniques, are to be understood as protocols and procedures found in an ordinary laboratory manual such as: Current Protocols in Molecular Biology, editors F. Ausubel et al., John Wiley and Sons, Inc. 1994, or Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989.

EXPERIMENTAL METHODS

Cloning and DNA construct

A human adipose tissue λ gt11 cDNA library (Clontech) was screened with a probe mixture corresponding to the conserved fork-head domain derived from: FOXC1,

FOXD1, FOXL1, and FOXA1. Hybridization was carried out at low stringency, *i.e.* 6xSSC at +60°C, post-hybridization washes at 0.5xSSC at +60°C. One of the positive recombinants harboring a 2.1 kb insert was subcloned and sequenced. A 5.4-kb *EcoRV-SmaI* fragment was excised from pBluescript II SK(+) vector containing the 5.4-kb promoter/enhancer of the mouse aP2 gene and ligated into the *EcoRV-SpeI* blunt site of pBluescript II SK(+) vector containing the 2.1 kb FOXC2 cDNA. A 7.6 kb *XhoI* blunt fragment containing the aP2 promoter/enhancer followed by the FOXC2 cDNA was excised from the above plasmid and ligated into the *EcoRV* site of the pCB6+ vector, which contains a polyadenylation signal from the human growth hormone gene. After these procedures the resulting 8.2-kb fragment, harboring the aP2-FOXC2 construct with polyadenylation signal, was flanked by the unique sites *NotI* and *AgeI*. The plasmid was sequenced over ligation sites.

Transgenic mice

Construct DNA (aP2-FOXC2), purified using Qiagen kit, according to manufacturer's instructions, was injected into the male pronucleus of (C57BL6 x CBA) F₁ zygotes, cultured over night and transferred to pseudopregnant females. Tg founder lines were back-crossed to C57BL6/J for four generations. Mice were feed a standard chow with 4% fat content. In experiments with high fat diet mice were fed either a chow with 58% fat or a control diet with 11.4% fat (on a caloric basis; Research Diets) for 7 weeks. High fat chow has a total energy content of 23.4KJ/g, control diet 12.6KJ/g.

Histology

Tissues were fixed over night in 4% paraformaldehyde in PBS at +4°C, dehydrated, embedded in paraffin, sectioned (6-8 µm) and stained with haematoxylin and eosin.

Serum and lipid analysis

Plasma insulin was determined radioimmunochemically with the use of a guinea pig anti-rat insulin antibody, ¹²⁵I-labeled porcine insulin as tracer and rat insulin as

standard (Linco). Free and bound radioactivity was separated by use of an anti-IgG (goat anti-guinea pig) antibody (Linco). The sensitivity of the assay is 17 pmol/l and the coefficient of variation is less than 3% at both low and high levels. Plasma glucose was determined with the glucose oxidase method and FFA was measured photometrically. Plasma glucagon was determined radioimmunochemically with the use of a guinea pig antiglucagon antibody specific for pancreatic glucagon, ¹²⁵I-labelled-glucagon as tracer, and glucagon standard (Linco). Free and bound radioactivity was separated by use of an anti-IgG (goat anti-guinea pig) antibody (Linco). The sensitivity of the assay is 7.5 pg/ml and the coefficient of variation is less than 9%. Blood levels of serum cholesterol and triglycerides were determined by fully enzymatic techniques ^{39,40}. Total body lipid was assessed using alcoholic hydroxide digestion with saponification of all lipids, neutralization, followed by enzymatic determination of glycerol.

Intravenous glucose tolerance test

The mice were anesthetized with an intraperitoneal injection of midazolam 0.4 mg/mouse (Hoffman-La-Roche) and a combination of fluanison (0.9 mg/mouse) and fentanyl 0.02 mg/mouse (Janssen). Thereafter, a blood sample was taken from the retrobulbar, intraorbital, capillary plexus in heparinized tubes, and D-glucose 1g/kg (British Drug Houses) was injected rapidly intravenously. New blood samples were taken after 1, 5, 20, and 50 minutes. Following immediate centrifugation at +4°C, plasma was separated and stored at -20°C or until analysis.

Northern blot

cDNA probes for mouse FoxC2, aP2, ADD-1/SREBP1, coxII, adipsin, $\beta_{1,3}$ -AR, GLUT4, IR, IRS1, and IRS2 were prepared by RT-PCR by use of first-strand cDNA from mouse epididymal fat poly(A)⁺ RNA. The PCR primers used to generate these probes were as follows:

- FoxC2: 5' primer, GCTTCGCCTCCTCCATGGGAA (SEQ ID NO:3) and 3' primer, GGTTACAAATCCGCACTCGTT (SEQ ID NO:4) (GenBank # Y08222).
- 5 aP2: 5' primer, CTC CTG TGCTGCAGCCTTTCTC (SEQ ID NO:5) and 3' primer, CGTAACTCACCACCACCAGCTTGTC (SEQ ID NO:6) (GenBank # M13261).
- ADD1/SREBP-1: 5' primer, GCCAACTCTCCTGAGAGCTT (SEQ ID NO:7) and 3' primer, CTCCTGCTTGAGCTTCTGGTT (SEQ ID NO:8) (GenBank # AB017337).
- 10 CoxII: 5' primer, CCATTCCAACCTGGTCTACAA (SEQ ID NO:9) and 3' primer, GGAACCATTTCTAGGACAATG (SEQ ID NO:10) (GenBank # J01420).
- Adipsin: 5' primer, CGAGGCCGGATTCTGGGTGGCCAG (SEQ ID NO:11) and 3' primer, TCGATCCACATCCGGTAGGATG (SEQ ID NO:12) (GenBank # X04673).
- 15 β_1 -AR: 5' primer, CGGCTGCAGACGCTCACCAA (SEQ ID NO:13) and 3' primer, CGCCACCAGTGCATGAGGAT ((SEQ ID NO:14) GenBank # L10084).
- β_2 -AR: 5' primer, GCTGCAGAAGATAGACAAAT (SEQ ID NO:15) and 3' primer, GGGATCCTCACACAGCAGTT (SEQ ID NO:16) (GenBank # X15643).
- 20 β_3 -AR: 5' primer, CTGCTAGCATCGAGACCTT (SEQ ID NO:17) and 3' primer, CGAGCATAGACGAAGAGCAT (SEQ ID NO:18) (GenBank # X60438).
- 25 GLUT4: 5' primer, CTCAGCAGCGAGTGACTGGGAC (SEQ ID NO:19) and 3' primer, CCCTGAGTAGGCGCCAATGAGG (SEQ ID NO:20) (GenBank # D28561).
- IR: 5' primer, GTAGCCTGATCATCAACATCCG (SEQ ID NO:21) and 3' primer, CCTGCCCATCAAACCTCTGTAC (SEQ ID NO:22) (GenBank # J05149).
- 30

IRS1: 5' primer, ATGGCGAGCCCTCCGGATACCG (SEQ ID NO:23) and 3' primer, CCTCTCCAACGCCAGAAGCTGCC (SEQ ID NO:24) (GenBank # X69722).

5 IRS2: 5' primer, GGATAATGGTGACTATACCGAGA (SEQ ID NO:25) and 3' primer, CTCACATCGATGGCGATATAGTT (SEQ ID NO:26) (GenBank # AF090738).

10 cDNA probes were radiolabeled with [α - 32 P]dCTP (3000 Ci/mmol) by the random labeling method. Total RNA from mice in each group was pooled, and aliquots of 12 μ g were separated on an agarose gel. The filters were hybridized with 32 P-labeled probe (10^6 cpm/ml) for 1 h at 62°C with QuikHyb solution (Stratagene) and washed with 0.1% SDS/0.1x SSC at +62°C for 3x20 min.

15 *Transfections and reporter gene analysis*

Non-confluent cultures of 3T3-L1 adipocytes were transfected with a CAT reporter (pCAT) driven by the human RI α proximal promoters upstream of the alternatively spliced 1a and 1b leader exons (nucleotides 1509 to 2470 GenBank # Y07641). To control transfection efficiency a pGL3control (Promega) luciferase-encoding vector was used. In cotransfections a *FOXC2* expression vector or vector void of insert was used. Transfections were carried out using lipofectamine (Gibco), followed by CAT and luciferase assays.

PKA immunoblotting and kinase activity

WAT and BAT were treated by a Polytron tissue homogenizer (3 x 15 s) and sonicated, on ice, in a buffer containing 10 mM potassium phosphate, pH 6.8, 150 mM sodium chloride, 1 mM EDTA, 10 mM CHAPS and protease inhibitors, and centrifuged (15,000 x g) to remove insoluble material. Protein concentrations were determined by Bradford assays (BioRad). For immunoblotting, 30 µg of protein was separated by 10% SDS-PAGE, transferred to PVDF membranes and incubated with anti-RI α and anti-RII β mAb. Primary antibodies were detected by HRP-conjugated anti-mouse IgG (Transduction Laboratories, 1:5000) and ECL (Amersham). PKA activity was measured using Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrate in the absence or presence of varying concentrations of cAMP. The low levels of activity not inhibited by PKI (2 µM) were subtracted to determine PKA-specific activity.

15

EXAMPLES OF THE INVENTION

EXAMPLE 1: Isolation of *FKHL14/FOXC2* from human abdominal fat tissue

5 To identify presumptive winged helix genes that are expressed in adipose tissue we screened a Human Fat Cell 5'-STRETCH plus cDNA Library (Clontech) using a low stringency hybridization strategy with a mix of cDNA probes corresponding to DNA-binding domains from different winged helix proteins, to cover the variation between different family members. By this technique, we were able to identify three different
10 forkhead genes, namely *FKHL5*, *FKHL14*, and *FKHL18*. *FKHL5* was known to be expressed exclusively in lung and placenta (Pierrou *et al.*, 1994) and expression in adipose tissue could not be detected as judged by Northern blot analysis (data not shown). *FKHL18* was identified as a novel member of the forkhead family (Cederberg *et al.*, 1997) having a very high homology with the forkhead motif of the
15 mouse gene *fkh-3* (Kaestner *et al.*, 1993). Sequence from outside the forkhead motif have not yet been published for *fkh-3* but with regard to the discrepancy of the expression patterns, with *FKHL18* being expressed in arterial vessel wall (aorta) and to a lower extent in kidney and *fkh-3* being expressed in a wide variety of tissues, they are presumed to constitute two different genes. We were not able to detect any
20 expression of *FKHL18* in adipose tissue (data not shown). The third gene that was identified, *FKHL14/FOXC2*, (Miura, N. *et al.* (1997) Genomics 41, 489-492) was shown to be expressed exclusively in human adult adipose tissue by Northern blot analysis (Fig. 3a). The mouse homologue of *FKHL14/FOXC2* is known as *Mfhl* (also known as *Foxc2*), and is expressed in dynamic patterns in the paraxial mesoderm of
25 the trunk and head, in the mesenchyme and endothelial cells of the branchial arches, and in many other sites in the embryo (Kaestner *et al.*, 1996; Miura *et al.*, 1993; Winnier *et al.*, 1997). It has been demonstrated by gene targeting experiments that *Mfhl* plays a crucial role during embryonic development. Homozygous null mutants died pre- and perinatally with multiple skeletal and cardiovascular defects, including
30 defects in the neurocranium and vertebral column, interruptions or coarctations of the aortic arch, and ventricular septal defects (Iida *et al.*, 1997; Winnier *et al.*, 1997).

Heterozygous mice were indistinguishable from wild-type and appeared healthy. The expression of *Mfhl* in adult tissues was restricted to WAT and BAT (Fig. 3).

5 EXAMPLE 2: *FKHL14/FOXC2* represses Ob gene expression *in vitro*

To be able to study the function of the *FKHL14/FOXC2* protein *in vitro* we prepared an expression construct containing the full length *FKHL14/FOXC2* cDNA sequence (SEQ ID NO: 1) inserted into the expression plasmid pCB6+ (Brewer, 1994). This
10 construct could then be used for transient transfection assays in mammalian cell culture systems to elucidate the action of *FKHL14/FOXC2* protein on promoters for different genes involved in adipocyte differentiation and development. To investigate the Ob promoter we used a Chinese hamster ovary (CHO) cell line (the promoter is known to be active in these cells). Transient co-transfections were performed using a
15 6.5-kb fragment of the Ob promoter coupled to the luciferase gene used as a reporter. A tenfold down-regulation of the activity of the Ob promoter was observed then co-transfected with *FKHL14/FOXC2* expression plasmid (Fig. 4).

20 EXAMPLE 3: Generation of transgenic mice overexpressing *FKHL14*

To elucidate whatever function *FKHL14/FOXC2* might have *in vivo* we decided to create transgenic mice overexpressing *FKHL14/FOXC2* in adipose tissue. To achieve overexpression of *FKHL14/FOXC2* in both white and brown adipose tissue, we
25 prepared a transgene construct (Fig. 5a) encoding *FKHL14/FOXC2* driven by a 5.4-kb DNA fragment containing the adipose-specific enhancer/promoter of the gene encoding adipocyte P2 (Ross *et al.*, 1990). Integration positive mice were identified using Southern blot analysis (Fig. 5b).

30 We studied three lines of transgenic mice (A, B, and C) that were derived from independent founders. Transgene expression, analyzed by Northern blot using a *FKHL14/FOXC2* (human specific) probe, was detected at high levels in both WAT

and BAT for all three founders. The three different founders had a slight difference in expression levels of the transgene in white fat, founder A having the highest and founder B the lowest, while the expression levels in brown fat were equal (Fig. 6). All of the transgenic mice used for the study were hemizygous for the transgene and from the second to fourth generation. The transgenic mice appeared normal at external inspection and the transgenic allele was propagated with the expected mendelian distribution. Fed a standard rodent chow diet (4% fat, 18.5% protein, and 55.7% sucrose) weight gain was studied for all founders from 4 weeks up to 6 months of age with no significant difference between nontransgenic and transgenic littermates noticed (data not shown).

EXAMPLE 4: WAT of *FKHL14/FOXC2* transgenic mice morphologically resembles BAT

The interscapular brown adipose tissue depot and the intrabdominal white adipose tissue were examined in 5-months-old mice (Fig. 7). Interscapular, transgenic mice had a greatly enlarged bilobed (dissected apart in the picture) fat pad with the “looks” of brown adipose tissue without the usual coverage of white adipose tissue (Fig. 7b), which normally have to be dissected to uncover the interscapular BAT depot (Fig. 7a). An exposed abdominal view of *FKHL14/FOXC2* transgenic mice revealed an obvious decrease of intrabdominal fat mass and also a distinct change in the appearance towards the brown adipose tissue phenotype (Fig. 7d), when compared to the large, pale lipid-storing intrabdominal fat pad of wild-type mice (Fig. 7c). The adipose tissue change in appearance and mass between transgenic and nontransgenic mice was very striking then the depots had been dissected out (Fig. 7e & f).

Histological analysis of BAT and WAT from 5-months-old mice revealed profound differences between transgenic and nontransgenic tissues. The majority of adipocytes in brown fat of the transgenic mice contained few markedly enlarged fat droplets (Fig. 8b), instead of small multilocular fat droplets typical for BAT (Fig. 8a). Adipocytes in intrabdominal white fat of the transgenic mouse showed heterogeneity of size, all of

them having a clearly reduced size (Fig. 8d). This was in contrast to WAT from wild-type mouse, which consisted of large adipocytes of uniform size filled with a large, unilocular lipid-storing vacuole (Fig. 8c).

5 To further examine the change of size of fat depots in transgenic mice, we compared the weights of intrabdominal WAT depots and interscapular BAT depots of 5-months-old wild-type females (n=3) and *FKHL14/FOXC2* transgenic littermates (n=3). In transgenic mice there was a decrease in weight of the intrabdominal WAT depot (~40%) whereas the interscapular BAT depot showed a marked increase (~7.5 times;
10 Fig. 9a). In consequence the ratio between weights of the intrabdominal fat depot and the interscapular brown fat depot was greatly decreased (Fig. 9b). No significant difference in body weight could be detected between transgenic and nontransgenic mice (Fig. 9c), nor could any difference in food consumption be observed then measured during a time period of two months (Fig. 9d).

15

EXAMPLE 5: Altered gene expression in *FKHL14/FOXC2* transgenic mice

Figure 10 shows an analysis of mRNA steady state levels in white and brown adipose tissue of wild-type mice and the three independent transgenic founders. In wild-type mice, the mRNA for uncoupling protein 1 (UCP1) was detectable only in BAT, but in the transgenic mice expression could be detected in WAT, in a dose responsive manner in proportion to the expression level of transgenic *FKHL14*. The WAT blot and BAT blot had different exposure times, 2 days and 30 minutes respectively. UCP2 seemed to have a tendency to be regulated in the opposite direction as compared with UCP1 in WAT of transgenic mice. The uncoupling protein expressed in skeletal muscle and BAT, UCP3, showed greatly decreased levels in transgenic BAT. The cytochrome c-oxidase subunit II (CoxII), a gene encoded by the mitochondrial genome, was used as a marker for density of mitochondria. The density of mitochondria in WAT and BAT from transgenic animals appeared to be elevated and to a lesser extent reduced, respectively. In wild-type mice, the mRNAs of β_1 - and β_3 -AR were much lower in WAT than in BAT. The *FKHL14/FOXC2* transgene abolished this discrepancy, raising these mRNAs selectively in WAT so that they became equal, or in the case of β_3 -AR even higher, compared to the levels in BAT. The β_2 -AR mRNA level was elevated in both WAT and BAT of transgenic mice, reaching levels not seen in nor WAT or BAT of wild-type littermates. White fat depots of transgenic animals exhibited profound increment in four of its mRNAs that are associated with fully differentiated adipocytes, that is, PPAR γ 2, C/EBP α , aP2, and adipsin. Furthermore PGC-1, a co-activator of PPAR γ 2, was upregulated in both WAT and BAT. The mRNA for ADD1/SREBP1 was elevated in WAT for all three founders. The transgenic animals demonstrated a reduction of the amount of leptin mRNA with the most distinct effect in BAT. Steady state levels of LPL mRNA appear to have increased slightly in WAT of transgenic mice. All investigated markers involved in insulin action were upregulated: the insulin receptor (InsR), IRS-1, IRS-2, and insulin-responsive glucose transporter-4 (GLUT4). The upregulation was most evident in WAT. GAPDH was used as a control to verify equal amounts of total RNA in the different lanes.

EXAMPLE 6: Triglyceride content is altered in *FKHL14/FOXC2* transgenic mice

Serum triglyceride content was analysed for 14-weeks-old wild-type males (n=4) and *FKHL14/FOXC2* transgenic littermates (n=4) fed ad libitum. The transgenic animals exhibited a ~60% reduction in serum triglyceride levels (Fig. 11).

Total body lipid content was assessed using alcoholic potassium hydroxide digestion with saponification of all fats, neutralization, and then enzymatic determination of glycerol (Triglyceride kit, Sigma) as described previously (Salmon and Flatt, 1985). The assay was carried out for 6-months-old wild-type males (n=4) and *FKHL14/FOXC2* transgenic littermates (n=4) fed ad libitum. The total body lipid content was reduced to 10% of the body weight for transgenic mice compared with the normal 30% of body lipid noted for wild-type mice (Fig. 12).

EXAMPLE 7: Lower blood glucose and more efficient glucose elimination in *FKHL14/FOXC2* transgenic mice

Nonfasting blood glucose levels were measured for 10-weeks-old wild-type males and females (n=5+5), and *FKHL14/FOXC2* transgenic littermates (n=5+5) fed ad libitum. The transgenic animals showed a significant 16% reduction in nonfasting blood glucose levels (Fig. 13a).

A glucose tolerance test was performed on 10-weeks-old wild-type males and females (n=5+5) and *FKHL14/FOXC2* transgenic littermates (n=5+5) fed ad libitum. Plasma glucose levels peaked at one minute after intravenous glucose administration, and thereafter plasma glucose levels returned to baseline levels within the 50-minutes study period. Transgenic mice displayed an enhanced glucose elimination, with plasma glucose levels significantly lower than wild-type littermates at 0, 20, and 50 minutes ($P<0.05$; $P<0.001$; $P<0.05$) (Fig. 13b). Data are means of both males and females as no difference between gender was noticed.

EXAMPLE 8: Increased insulin sensitivity in *FKHL14/FOXC2* transgenic mice

5 Non-fasting plasma insulin levels in *FKHL14/FOXC2* transgenic mice were analyzed using the same group of animals as was used for blood glucose measurements. The concentration of plasma insulin in *FKHL14/FOXC2* transgenic mice before the start of the glucose tolerance test were reduced to ~50% of the levels registered for wild-type littermates (Fig. 14a). The rapid intravenous injection of glucose (1 g/kg) raised
10 plasma insulin levels 4-fold in wild-type mice and 10-fold in *FKHL14/FOXC2* transgenic littermates after one minute (Fig. 14c). Thereafter, plasma insulin levels rapidly returned toward baseline values observed before the glucose load, with transgenic mice having significantly lower levels at 20 and 50 minutes ($P<0.05$; $P<0.05$) (Fig. 14b). Data are means of both males and females as no difference
15 between gender was noticed.

In mice on a high fat diet, there are significantly lower weight gains in transgenic mice as compared with wt. In females the weight gain is 39% ($p<0.02$; Fig. 16a) lower as compared with wt females and for males the difference is 21% ($p<0.03$; Fig.
20 16b). These findings highlight *FKHL14/FOXC2*, not only as a gene of importance for adipose tissue distribution, morphology and gene expression profile, but also, more importantly, as a major regulator of general lipid and glucose metabolism including protection against diet induced weight gains.

25

EXAMPLE 9: Interaction trap / two-hybrid system

In order to assay for polypeptides interacting with the *FKHL14/FOXC2* polypeptide, the interaction trap/two-hybrid library screening method can be used. This assay was
30 first described in Fields & Song (1989) Nature 340, 245-246. Kits are available from Clontech, Palo Alto, CA (Matchmaker Two-Hybrid System 3).

A fusion of an *FKHL14/FOXC2* nucleotide sequence and the yeast transcription factor GAL4 DNA-binding domain (DNA-BD) is constructed in an appropriate plasmid (e.g. pGBKT7) using standard subcloning techniques. Similarly, a GAL4 active domain (AD) fusion library is constructed in a second plasmid (ie. pGADT7) from cDNA of potential binding proteins. The DNA-BD fusion construct is verified by sequencing, and tested for autonomous reporter gene activation and cell toxicity, both of which would prevent a successful two-hybrid analysis. Similar controls are performed with the AD/library fusion construct to ensure expression in host cells and lack of transcriptional activity. Yeast cells are transformed (ca. 10^5 transformants/mg DNA) with both the DNA-BD and library fusion plasmids according to standard procedures. *In vivo* binding of DNA-BD/(FKHL14/FOXC2) with AD/library proteins results in transcription of specific yeast plasmid reporter genes (i.e., lacZ, HIS3, ADE2, LEU2). Yeast cells are plated on nutrient-deficient media to screen for expression of reporter genes. Colonies are dually assayed for β -galactosidase activity upon growth in Xgal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) supplemented media (filter assay for β -galactosidase activity is described in Breeden, et al. (1985) Cold Spring Harb. Symp. Quant. Biol. 50, 643). Positive AD-library plasmids are rescued from transformants and reintroduced into the original yeast strain as well as other strains containing unrelated DNA-BD fusion proteins to confirm specific (FKHL14/FOXC2)/library protein interactions. Insert DNA is sequenced to verify the presence of an open reading frame fused to GAL4 AD and to determine the identity of the binding protein.

EXAMPLE 10: Mobility shift DNA-binding assay

A gel electrophoresis mobility shift assay, according to standard procedures, can rapidly detect specific protein-DNA interactions. Probe DNA (<300 bp) is obtained from synthetic oligonucleotides, restriction endonuclease fragments, or PCR fragments and end-labeled with ^{32}P . An aliquot of purified FKHL14/FOXC2 (ca. 15 μg) or crude FKHL14/FOXC2 extract (ca. 15 ng) is incubated at constant temperature (in the range 22–37°C) for at least 30 minutes in 10–15 μl of buffer (i.e. TAE or TBE, pH 8.0–8.5) containing radiolabeled probe DNA, nonspecific carrier DNA (ca. 1 μg), BSA (300 $\mu\text{g}/\text{ml}$), and 10% (v/v) glycerol. The reaction mixture is then loaded onto a polyacrylamide gel and run at 30–35 mA until good separation of free probe DNA from protein-DNA complexes occurs. The gel is then dried and bands corresponding to free DNA and protein-DNA complexes are detected by autoradiography.

15

EXAMPLE 11: Reporter gene assay to identify modulating compounds

Reporter gene assays are well known as tools to signal transcriptional activity in cells. (For a review of chemiluminescent and bioluminescent reporter gene assays, see Bronstein et al. (1994) *Analytical Biochemistry* 219, 169–181.) For instance, the photoprotein luciferase provides a useful tool for assaying for modulators of FKHL14/FOXC2 activity. Cells (e.g., CHO cells or COS 7 cells) are transiently cotransfected with both a FKHL14/FOXC2 expression construct and a reporter construct which includes a gene for the luciferase protein downstream from a transcription factor binding site. Agonist binding to FKHL14/FOXC2 results in expression of the luciferase gene. Luciferase activity may be quantitatively measured using e.g. luciferase assay reagents that are commercially available from Promega (Madison, WI). Differences in luminescence in the presence versus the absence of a candidate modulator compound are indicative of modulatory activity.

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